

AMENDMENTS TO THE SPECIFICATION

Please replace the previously amended paragraph bridging pages 8 and 9 of the specification, with the following amended paragraph:

To achieve the functional objective of an initially time-delayed PG activation kinetics by the hybrid SK derivatives, our design utilizes the fusion of selected regions of the FBDs of human fibronectin or its homologous sequences present in other proteins with SK (or its partially truncated forms) at strategically useful points so as to kinetically hinder the initial interaction of SK with PG necessary to form the 1:1 stoichiometric activator complex. It is known that of the 414 residues constituting native SK only the first 15 residues and the last 31 residues are expendable, with the resultant truncated polypeptide being nearly as active as the native full-length protein in terms of PG activation ability (~~Young, K.C. et al., (1995) J. Biol. Chem. 270:29601-29606; Jackson K.W., Malke H., Ferretti J.J., and Tang J (1986) Biochemistry 25:108-114~~ Jackson, K. W. and Tang, J. (1982) Biochemistry 21:6620). Further truncation at either end results in drastic decrease in the activity associated with the molecule (Malke, H., Roe, B., and Ferretti, J. J. (1987) In: *Streptococcal Genetics*. Ferretti, J. J., and Curtis, R. III [Ed.] Proc. American Society for Microbiology., Wash. D.C. p. 143). It has been demonstrated that SK interacts with PG through at least two major loci, mapped between residues 16-51 and 230-290 (Nihalani, D., Raghava, G. P. S., and Sahni, G., 1997, Prot. Sci. 6:1284), and probably also the region in and around residues 331-332 (Lin, F. L., Oeun S., Houn, A., and Reed, G. L., 1996, Biochemistry 35:16879); in addition, the sequences at the C-terminal ends, especially before the last 30-32 residues of the native sequence (Kim. I. C., Kim, J. S., Lee, S. H., and Byun, S. M.

1996, *Biochem. Mol. Bio. Int.* 40:939. Lee, S. H., Jeong, S. T., Kim, I. C. and Byun S. M. 1997 *Biochem. Mol. Bio. Int.* 41:199. Fay, W. P., Bokka, L. V., 1998, *Thromb. Haemost.* 79:985) are important in generating the activator activity associated with the complex. Since a primary consideration in designing the SK-FBD chimeras was the ~~the~~ engineering of a decreased, or kinetically slowed, initial PG activation rate, we reasoned that either the C- or N-termini (or both, together) could be utilized to bear the FBDs in the hybrid structures, and that the presence of such extra domains in SK, either full-length or already truncated to the most functionally relevant regions of human fibronectin that can independently bind fibrin under physiological conditions (detailed earlier) *and* would also suitably retard and/or delay the PG activation rates observed normally with native SK and PG by interfering in the interactions of SK with PG to generate a functional activator complex. If the polypeptide in between these two distinct parts constituting the chimera were sufficiently flexible, proteolytic scission in this region would then result in the removal of the retarding/inhibiting portion (FBD component) from the SK-FBD hybrid and lead to a burst of PG activation after an initial delay. This proteolysis could be mediated by the small amounts of endogenous plasmin generated in the vicinity of the pathological clot by intrinsic plasminogen activator/s of the system, such as TPA, urokinase etc already present in the circulatory system. Indeed, this premise was borne out by experimentation, which showed that the lag times of PG activation by the SK-FBD chimeras disclosed in this invention were directly governed by plasmin-mediated proteolysis of the hybrid proteins leading to the liberation of the FBD portion from the SK-FBD followed by rapid PG activation by the SK. The direct implication of this functional property in a plasminogen activator is that once

injected into the body, the protein could then traverse in an inactive state through the circulatory system and bind to the pathological clot by virtue of the fibrin affinity imparted by the fibrin binding domains thereby obviating or minimizing systemic PG activation. Thus, if the thrombolytic agent traverses the circulation prior to this activation (which is known to require 3-5 minutes in the human circulation), the fibrin affinity in the chimera would allow it to bind to the clot, thereby helping to localize the PG activation in and around the immediate vicinity of the thrombus.

Please replace the Table at page 57 of the specification with the following reformatted Table:

Table 1

Steady-state kinetic parameters for HPG activation by SK and SK-FBD hybrid proteins*

Activator protein	k_{plg} (μ M)	Maximal activity#	Lag (min)
nSK	0.14 ± 0.02	100.0	1.0
Met-SK	0.18 ± 0.01	95.5 ± 5	2.0
SK-FBD(4,5)	0.15 ± 0.02	52 ± 4	10.0
SK-FBD(1,2)	0.18 ± 0.03	58 ± 5	10.5
FBD(4,5)-SK	0.16 ± 0.02	65 ± 4	8.0
FBD(4,5)-SK-FBD(4,5)	0.20 ± 0.03	45 ± 4	18.0

Please replace the first paragraph of page 55 with the following amended paragraph:

The proteins prepared in Example 7, above, as well as native and Met-SK (as controls) were examined with respect to their PG activation kinetics. This essentially entailed the study of the time-course of PG activation by the various SK/FBD chimeras and the determination of their steady-state kinetic constants for PG activation. A one-stage assay method was used to measure the activation of HPG; reference in this context may be made to several publications in the literature e.g., Shi, G. Y., Chang, B. I., Chen, S. M., Wu, D. H. and Wu H. L., 1994, *Biochem. J.* 304:235; Wu, H. L., Shi, G. Y., and Bender, M. L., 1987, *Proc. Natl. Acad. Sci.* 84: 9292; Wohl, R. C., Summaria, L., and Robbing, K. C., 1980, *J. Biol. Chem.* 255: 2005; Nihalani, D., Raghava, G. P. S., Sahni, G., 1997, *Prot. Sci.* 6:1284). Briefly, it involved the addition of the activator proteins to be studied in a small aliquot (~5 ul) into 100 ul-volume microvette containing 1 uM of HPG in assay buffer (50 mM Tris-Cl buffer, pH 7.5, containing 0.5 mM chromogenic peptide substrate and 0.1 M NaCl). The protein aliquots were added after addition of all other components into the cuvette and bringing the spectrophotometric absorbance to zero. The change in absorbance at 405 nm was then measured as a function of time in a Shimadzu UV-160 model spectrophotometer (FIG.). While SK showed a rapid PG activation kinetics, the kinetics for SK-FBD chimeric protein [shown for SK-FBD(4,5) in FIG.] displayed a characteristic lag, or delay, in the initial phase of the rate of PG activation that was clearly different from the rates seen with SK. This property viz., initial delay in HPG activation, as well as its magnitude, was largely

independent of the amount of the chimeric protein employed in the assay, as well as the concentration of HPG in the reaction. Another notable feature was that the lag-times associated with the different chimeric proteins under the same conditions. In the case of SK-FBD(1,2) and SK-FBD(4,5) the lag period corresponded to 10-12 min, for FBD(4,5)-SK 7-8 min, and 20-25 min in case of FBD(4,5)-SK-FBD(4,5). Under the same conditions (~1 uM HPG, 1-2 nM of protein), native SK or Met-SK displayed very little lag period (i.e. less than 1 min duration) during PG activation.

Please replace the legend for Fig. 18, at page 13 of the specification, with the following amended legend:

FIG. 18. Schematic flow diagram for cloning of SK-FBD(1,2) hybrid gene in pBluescript, to obtain [pSKMG400-FBD(1,2)]~~[pSKMG400-FBD(1,2)]~~.

Please replace the previously amended legend for Fig. 7 at page 12 of the specification, with the following original legend:

FIG. 7. Restriction enzyme map of DNA encoding the five N-terminally located FBDs of human fibronectin.

Please replace the Abstract with the following amended Abstract:

The invention disclosed herein provides clot~~Clot~~-specific streptokinase proteins possessing altered plasminogen characteristics, including enhanced fibrin selectivity. The kinetics of plasminogen activation by these proteins are distinct from those of natural streptokinase, in that there is a temporary delay or lag in the initial rate of catalytic conversion of plasminogen to plasmin. Also disclosed are processes for preparing the proteins.